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Amplification of the *aroA* Gene from *Escherichia coli* Results in Tolerance to the Herbicide Glyphosate

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The predominant cellular target of the herbicide glyphosate is thought to be the enzyme 5-enolpyruvylshikimate-3-phosphoric acid synthase (EPSP synthase). As a means of biologically testing this finding, we cloned a segment of DNA from *Escherichia coli* that encodes this enzyme. Clones carrying the gene for EPSP synthase were identified by genetic complementation. Cells that contain a multicopy plasmid carrying the EPSP synthase gene overproduce the enzyme 5- to 17-fold and exhibit at least an 8-fold increased tolerance to glyphosate. These experiments provide direct biological evidence that EPSP synthase is a major site of glyphosate action in *E. coli* and that, in an amplified form, it can serve as a selectable glyphosate resistance marker.

Glyphosate (*N*-[phosphonomethyl]glycine) is a potent, postemergent herbicide that is toxic to most actively growing plants and many bacteria but not to insects or vertebrates (19; D. D. Baird, R. P. Upchurch, W. B. Homesley, and J. E. Franz, Proc. North Cent. Weed Control Conf. **26**:64, 1971). This unique property suggests that the site of glyphosate action is in a biochemical pathway peculiar to plants and bacteria.

In 1972, Jaworski (10) reported the first attempt to identify the site of action. He found that glyphosate affected aromatic amino acid biosynthesis in a bacterium (*Rhizobium japonicum*) and duckweed (*Lemna gibba*). This conclusion was based on the reversal of glyphosate-induced growth inhibition by the addition of the aromatic amino acids phenylalanine and tyrosine. Similar experiments and results were reported by Gresshoff (9) in a bacterium (*Escherichia coli*), an alga (*Chlamydomonas reinhardii*), a whole plant (*Arabidopsis thaliana*), and plant cell cultures (carrot and soybean). These biological studies all suggested a glyphosate effect on aromatic amino acid biosynthesis, but they did not pinpoint the enzyme in the pathway that was involved.

The enzymatic step inhibited by glyphosate has been subsequently identified by Amrhein and his colleagues (1, 22). These workers reported specific inhibition of the 5-enolpyruvylshikimate-3-phosphate synthase (EPSP synthase) reaction (Fig. 1) in soluble extracts of *Aerobacter aerogenes* treated with glyphosate. This enzyme catalyzes the sixth reaction in the shikimic acid pathway. Its product is required for chorismic acid synthesis. A block here would prevent synthesis of phenylalanine, tyrosine, and tryptophan consistent with the reversal of glyphosate action cited above.

These results suggest that glyphosate-tolerant cells may be obtained either by alteration of the target EPSP synthase protein, so that the herbicide binds to the enzyme less efficiently relative to the substrate, or by amplification of the EPSP synthase enzyme. In either case, more herbicide would be required to effect complete inhibition. Recombinant DNA techniques provide a means of testing the amplification hypothesis with *E. coli* as a model system.

In *E. coli* the genes for aromatic amino acid biosynthesis are distributed throughout the genome (15). Mutants defective in EPSP synthase, called *aroA*, are located at 20 min on the standard *E. coli* genetic map (2) and require aromatic amino acids for growth. The use of *aroA* mutants allows specific isolation of the EPSP synthase gene by genetic complementation. The EPSP synthase gene is expressed constitutively (24). Therefore, cells that carry the *aroA* gene in a multicopy plasmid will oversynthesize EPSP synthase. If this enzyme is the glyphosate target, the cells will be more tolerant. The experiments described in this report show that amplification of the *E. coli aroA* gene results in oversynthesis of EPSP synthase and produces increased tolerance to glyphosate.

MATERIALS AND METHODS

Bacteria and plasmids. A list of the bacterial strains and plasmids used in this study appears in Table 1.

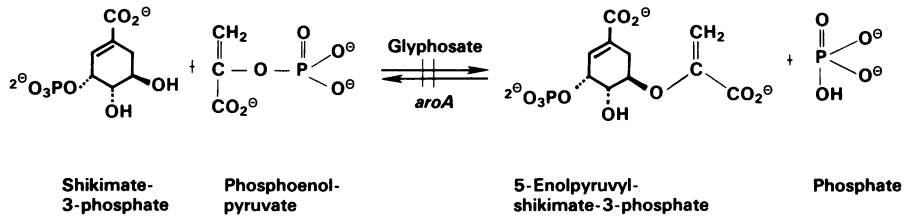

FIG. 1. Enzymatic reaction catalyzed by EPSP synthase showing the structures of the reactants and products. The lines across the arrows refer to the blockage of this step in aromatic amino acid biosynthesis by the *E. coli aroA* mutation or by the herbicide glyphosate.

Those constructed as a part of this study are described in the text.

Media. Luria broth (LB) contained 10 g of tryptone (Difco Laboratories, Detroit, Mich.), 5 g of yeast extract (Difco), and 5 g of NaCl per liter of deionized water. The pH was adjusted to 7.4 with 2 N NaOH before autoclaving. For plates, Bacto-Agar (Difco) was added to 1.8%. M9 minimal medium was prepared as described by Miller (13). VB minimal medium was prepared as described by Vogel and Bonner (25). Glucose and thiamine were added after autoclaving to both M9 and VB at 0.4% and 10 μg/ml final concentrations. Solid minimal salts plates were made by the addition of Bacto-Agar to 1.5%. When required, supplements or antibiotics were added at the following final concentrations from filter sterilized stocks: ampicillin (Amp), 200 μg/ml for LB plates or 50 μg/ml for minimal salts plates; proline (Pro), 200 μg/ml; tryptophan (Trp), tyrosine (Tyr), histidine (His), or phenylalanine (Phe), 100 μg/ml; kanamycin sulfate (Kan), 40 μg/ml; tetracycline (Tet) 20 μg/ml. For optimal growth of *aroA* cells in minimal media, *p*-hydroxybenzoate and *p*-aminobenzoate were added at a final concentration of 20 μg/ml. A stock solution of 100 mM glyphosate (analytical grade; Monsanto Co., St. Louis, Mo.) was prepared in deionized water, adjusted to pH 7.6 with 2 N NaOH, and filter sterilized. All supplements or antibiotics were added after the medium had cooled to 50°C.

Preparation of DNA. Phage λ DNA was prepared by heat induction of lysogens, banding in CsCl gradients, and phenol extraction as described by Miller (13). Plasmid DNA was prepared after chloramphenicol amplification from cleared lysates as described by Clewell (5), except that 0.2% Triton X-100 was used for lysis.

Restriction endonucleases. All restriction endonucleases were obtained from New England Biolabs (Beverly, Mass.) and used according to the supplier's instructions. For nomenclature and site specificity, see reference 18.

Ligation of DNA. Ligations were carried out in 50-μl volumes containing 50 mM Tris-hydrochloride (pH 7.6), 10 mM dithiothreitol, 10 mM $MgCl_2$, 1 mM ATP (pH 7), and 300 to 450 U of T4 DNA ligase (New England Biolabs). The DNA concentration varied from 30 to 300 μg/ml. Incubation time was 2 to 16 h at 10°C.

Gel electrophoresis of DNA. DNA was analyzed by electrophoresis in horizontal 0.7% agarose gels in buffer (40 mM Tris base, 20 mM sodium acetate, 1 mM disodium EDTA; pH adjusted to 8.0 with glacial acetic acid) containing 0.1 μg of ethidium bromide per ml. Electrophoresis was performed at 40 to 100 V for 2.5 to 4 h. Gels were photographed with Polaroid type 52 film after visualization of the bands on a C63 Transilluminator (UV Products, San Gabriel, Calif.). Sizes of restriction fragments were determined from their mobilities relative to *Hin*dIII fragements of phage λ DNA (7).

Construction of λKR2-*E. coli* *Hin*dIII library. The integration-proficient vector λKR2 (S. Rogers, unpublished data) is a λ*imm*^{21} *ninR5 Sam7* *Hin*dIII replacement vector with *Hin*dIII sites at coordinates 0.40 and 0.58 from the left end. The hybrid library was constructed by using *E. coli* KL16 DNA (10- to 15-kilobase [kb] *Hin*dIII fragments) and purified λKR2 *Hin*dIII arms as described by Maniatis et al. (12).

Bacteriophage λ transductions. Cells were grown for transduction in LB broth–10 mM $MgSO_4$–0.2% maltose to 2×10^8 cells per ml. The cells were pelleted, suspended in ½ volume 10 mM Tris-hydrochloride (pH 8)–10 mM $MgSO_4$, and starved by aeration at 30°C for 1 h. These cells may be stored at 4°C for 1 month without loss of viability. Phage λ-*E. coli* hybrids in 0.1 ml of 10 mM Tris-hydrochloride (pH 8)–10 mM $MgSO_4$ were mixed with 0.2 ml of starved cells and incubated at 30°C for 30 min. After the addition of 2 ml of LB broth containing 20 mM sodium citrate, the cells were incubated at 30°C with aeration for 1 h. The cells were washed three times by centrifugation and resuspension in 2 ml of VB salts. Final suspension was in 0.1 ml of VB salts. The cells were spread on a VB plate and incubated at 30°C. Bacteriophage titers were determined by plating on a layer of LE392 cells by standard methods (13).

Transformation of *E. coli* cells. The procedure for introduction of plasmid DNA into cells was essentially that described by Taylor et al. (23). Briefly, cells were grown in LB broth to 2×10^8/ml, centrifuged, resuspended in ½ volume of 50 mM $CaCl_2$–1 mM Tris-hydrochloride (pH 8), and kept at 0°C for 20 min. The cells were then pelleted and resuspended in 1/10 the original culture volume of $CaCl_2$-Tris. Calcium-shocked cells (0.2 ml) were added to 0.1 ml of 10 mM Tris-hydrochloride (pH 8)–10 mM $MgCl_2$ containing plasmid DNA (0.01 to 1 μg) and incubated for 60 min on ice. The mixture was transferred to 37°C for 3 min and then 25°C for 10 min. After the addition of 2 ml of LB broth, the cells were incubated without aeration at 37°C for 1 h. The cells were pelleted and resuspended in 0.1 ml of LB broth and spread on selective plates at

TABLE 1. Strains used

Strain	Relevant properties[a]	Source or reference
E. coli K-12		
LE392(ED8656)	*supE supF*	L. Enquist
594	*sup*^{0} *rpsL* prototroph	F. Stahl
AB1321	*his-4 proA2 aroA2*	CGSC[b]
KL16	*relA sup*^{0}	B. Weiss
JC10240	Hfr PO45 *srlC300*::Tn*10 recA56*	(6)
AB2829	*aroA354* prototroph	CGSC
SR484	AB2829 *rpsL* by P1 transduction from 594	This paper
SR486	SR484 *aroA*$^+$ by P1 transduction from 594	This paper
SR485	SR484 *aroA354 srlC300*::Tn*10 recA56*	This paper
SR488	SR486 *aroA*$^+$ *srlC300*::Tn*10 recA56* by mating with JC10240	This paper
Plasmids		
pBR322	mini-ColE1 Amp^r Tet^r	(3)
pBR327	Deletion derivative of pBR322 Amp^r Tet^r	(21)
pKC7	mini-ColE1 Amp^r Kan^r	(17)

[a] Genetic nomenclature: *E. coli*, reference 2; phage λ, reference 8; *Hin*dIII, sites of cleavage for *Hin*dIII restriction endonuclease; Amp^r, Tet^r, and Kan^r, genes for Amp, Tet, and Kan resistance, respectively.
[b] CGSC, *E. coli* Genetic Stock Center, Yale University School of Medicine, New Haven, Conn.

37°C. When the *aroA* gene was being selected, the cells were washed in VB salts as described above.

Measurement of growth in the presence of glyphosate. Cells were grown in freshly prepared 5-ml portions of M9 Amp containing appropriate amounts of 100 mM glyphosate in 15-ml colorimeter tubes. The pH of M9 is 6.9. The addition of glyphosate lowered the pH as follows: 0.1 to 1.0 mM, 6.9; 5 mM, 6.8; 10 mM, 6.7; 20 mM, 6.5; and 40 mM, 5.9. It was not necessary to adjust the pH after the addition of glyphosate, but it should be noted that the pH is lowered at high concentrations of the herbicide. This could lead to an underestimate of the growth rate of cells at the highest glyphosate concentration. Tubes were inoculated with 0.4 ml of an overnight culture grown in M9 Amp and incubated on a roller at 37°C. Cell density was determined as the optical density at 540 nm.

Assay of EPSP synthase. EPSP synthase activity was assayed by direct measurement of EPSP generated from 3-phosphate-[^{14}C]shikimate and phospho(enol)-pyruvate by a procedure described elsewhere (E. S. Sharps, manuscript in preparation). Cells for assay were grown in LB with Amp (200 μg/ml) and aromatic amino acids to an optical density of 0.35 at 540 nm. Unless otherwise stated, all of the subsequent steps were carried out at 0 to 4°C. The cells were collected by centrifugation, suspended in a buffer consisting of 50 mM sodium acetate (pH 5.25), 2.5 mM KF, and 5 mM $MgCl_2$ at 1 g of cells per 4 ml of buffer, and disrupted by sonication. Cellular debris was removed by centrifugation, and portions of the supernatant were used for enzymatic assay and protein determinations. The units of enzyme activity are nanomoles of EPSP produced per minute per milligram of protein at 37°C. The assay is linear in the range of 0.5 to 5 U of enzyme activity and is inhibited by glyphosate.

Chemicals. Shikimate-3-phosphate was prepared by the method of Knowles and Sprinson (11).

Protein determination. Protein concentrations were measured by the microbiuret method (14) with bovine serum albumin (Sigma; fraction V) as a standard.

Containment. All described experiments involving recombinant DNA are exempt from the National Institutes of Health guidelines but were carried out with appropriate microbiological safety practices.

RESULTS

Identification and characterization of λKR2p*aroA* transduction phage. A bacteriophage λ carrying the *E. coli aroA* gene specifying EPSP synthase was isolated from a library of *E. coli* KL16 *Hin*dIII restriction fragments carried in the vector λKR2. Strain AB1321 was transduced with the pooled library, and *aroA*$^+$ transductants were selected by their ability to form colonies on VB His Pro plates at 30°C. One colony out of several hundred was picked and grown in LB at 30°C, and phage were obtained by temperature induction (titer, 5×10^8 PFU/ml). These phage, λKR2p*aroA*, were used to retransduce AB1321 to *aroA*$^+$ (multiplicity of infection, 0.01) to confirm the presence of the *aroA* gene in the phage. The high titer of phage and high frequency of transduction obtained strongly suggested that the entire *aroA* gene is present in the phage and that *aroA*$^+$ transductants were not a result of recombination between an incomplete, phage-borne *aroA* segment with the chromosomal *aroA* gene to generate a functional EPSP synthase gene. Analysis of λKR2p*aroA* DNA by *Hin*dIII digestion and electrophoresis showed that the *aroA* phage carried a single *Hin*dIII insert of 11,000 base pairs.

In lysogens, λ phage is present at only one copy per chromosome. To increase the copy number of the *aroA* gene and the EPSP synthase enzyme, the *aroA Hin*dIII fragment was trans-

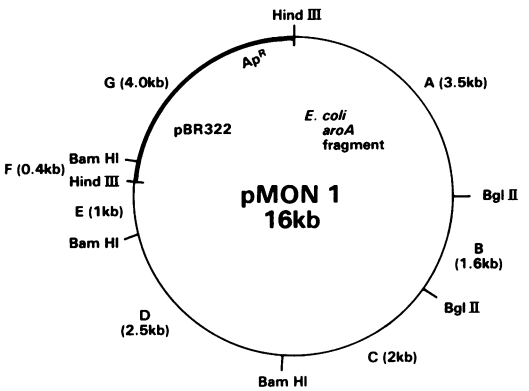

FIG. 2. Restriction endonuclease map of pMON1, a pBR322-*E. coli aroA* plasmid. The letters arbitrarily designate fragments of the plasmid DNA. The numbers in parentheses refer to the fragment size in kb. Ap^R is the Amp resistance determinant carried by pBR322.

ferred to plasmid pBR322, which is present in cells at 20 to 50 copies per chromosome (16).

Construction and characterization of pMON1, a pBR322 derivative carrying the *aroA* gene. Phage λKR2p*aroA* DNA (5 μg) was digested with *Hin*dIII and mixed with 0.2 μg of *Hin*dIII-cleaved pBR322 DNA, treated with T4 DNA ligase, and used to transform AB1321 cells. Transformants were selected on LB Amp plates at 37°C. Cells resistant to Amp were picked to VB His Pro Amp, LB Tet, and LB Amp plates to score the *aroA* phenotype and Tet resistance (3). One colony out of 60 was $aroA^+$ Tet^s. The other colonies contained religated pBR322 vector. Plasmid was purified from this cell, and the DNA was characterized by restriction endonuclease digestion. A restriction endonuclease cleavage map of this plasmid, pMON1, is shown in Fig. 2. The 11-kb *Hin*dIII fragment was present in pMON1. This *Hin*dIII insert contained no sites for the enzymes *Sal*I, *Xho*I, *Xba*I, *Eco*RI, *Sac*I, or *Sma*I. There were two sites for *Bam*HI and two sites for *Bgl*II. Their positions were determined by various double digests and appear in Fig. 2.

Our restriction endonuclease map of the 11-kb *Hin*dIII *aroA* fragment is identical to two independently derived restriction endonuclease maps of DNA from the 20-min region of the *E. coli* K-12 genetic map. The published maps are of a λ*aspC* phage (4) and a λp*serC* phage (20). These two genes map near the *aroA* locus (2). The identity of these restriction endonuclease maps provides further evidence that the 11-kb *Hin*dIII fragment originates in the *aroA* region of the *E. coli* chromosome.

Transfer of pMON1 to the prototrophic *E. coli* strain 594. *E. coli* cells are sensitive to glyphosate when grown in minimal medium. Growth inhibition is reversed by the addition of the aromatic amino acids phenylalanine, tyrosine, and tryptophan (9). To test whether amplification of *aroA* could confer tolerance to glyphosate, we needed to compare the growth of cells carrying pMON1 to that of cells carrying pBR322. However, this experiment cannot be done in AB1321 because the control AB1321/pBR322 cell requires aromatic amino acids for growth, and addition of the aromatic amino acids negates the effect of glyphosate on growth. Therefore, pMON1 and pBR322 were used to transform the prototrophic *E. coli* 594 to Amp resistance. *E. coli* 594/pBR322 formed normal colonies on LB Amp plates. In contrast, *E. coli* 594/pMON1 colonies grew extremely slowly and were irregular in shape, indicating segregation of the plasmid. This probably was due to the oversynthesis of a gene product specified by the 11-kb *Hin*dIII fragment. Indeed, reexamination of AB1321 cells carrying the pMON1 plasmid revealed the same slow growth and irregular colony morphology. Since subcloning of smaller segments of the *Hin*dIII fragment might separate this inhibitory function from the *aroA* gene, deletion derivatives of pMON1 were constructed as described below.

Localization of the *aroA* gene by construction of deletion derivatives. Plasmid pMON1 (2 μg) was cleaved with either *Bam*HI or *Bgl*II, treated with T4 DNA ligase, and used to transform AB1321 to Amp resistance on LB Amp plates at 37°C. These manipulations should result in a *Bam*HI deletion of fragments D, E, and F (Fig. 2) or a *Bgl*II deletion of the 1.6-kb B fragment (Fig. 2). Examination of the transformation plates revealed that the *Bam*HI deletion colonies were uniformly small, whereas the *Bgl*II deletion colonies were normal size. Testing of both types of colonies on VB His Pro Amp plates showed that all of the colonies were $aroA^+$. Therefore, *aroA* oversynthesis is not detrimental to cell growth, and deletion of the 1.6-kb *Bgl*II B fragment removes an inhibitory function from the plasmid. The *Bgl*II deletion plasmid, pMON2, is shown in Fig. 3.

Since deletion of the 1.6-kb *Bgl*II fragment leaves *aroA* intact, the coding sequence must lie entirely within either the 3.5-kb *Hin*dIII-*Bgl*II fragment or the 5.5-kb *Hin*dIII-*Bgl*II fragment of pMON2. To determine which fragment carried *aroA*, pMON2 DNA (2 μg) was digested with both enzymes and mixed with 0.2 μg of plasmid pKC7 (17) DNA treated similarly. The DNA was joined with T4 DNA ligase and used to transform AB1321 to Amp resistance. Insertion of a *Hin*dIII-*Bgl*II fragment in pKC7 results in the loss of Kan resistance carried by this plasmid.

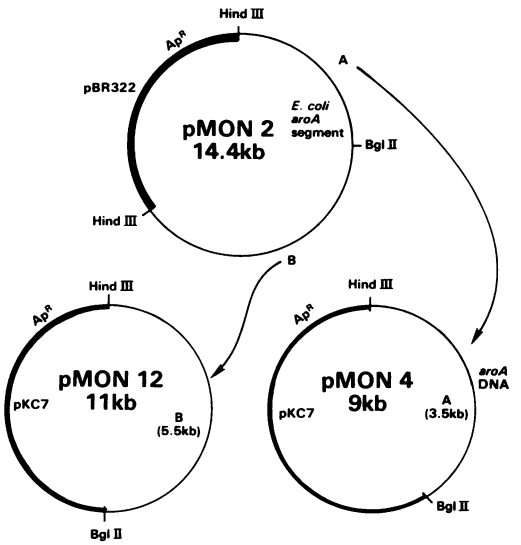

FIG. 3. Structure of plasmid pMON2 and construction of pMON4 and pMON12. The letters A and B refer to the two *Hin*dIII-*Bgl*II fragments produced by digestion with these restriction endonucleases. Ap^R is the Amp resistance determinant carried by pBR322 and pKC7.

Amp-resistant cells were picked to VB His Pro Amp, LB Kan, and LB Amp plates. The Amp-resistant, Kan-sensitive colonies fell into 2 classes: *aroA*$^+$ and *aroA*. Plasmid was prepared from one of each and analyzed by restriction endonuclease digestion. The *aroA* plasmid, pMON12, carried the 5.5-kb *Hin*dIII-*Bgl*II B fragment. The *aroA*$^+$ plasmid carried the 3.5-kb *Hin*dIII-*Bgl*II A fragment and was named pMON4 (Fig. 3).

Plasmids that complement the *aroA* defect synthesize increased levels of EPSP synthase. Enzymatic assay of extracts prepared from cells carrying pMON2, pMON4, pMON12, and pBR327 confirmed that plasmids which complement the *aroA* defect encode EPSP synthase. Assays were performed with extracts of SR485 cells (*aroA354 recA56*) carrying the plasmids. As a measure of the single-copy level of EPSP synthase, extracts of the congenic *aroA*$^+$ *recA56* strain, SR488, were also assayed. The results appear in Table 2.

As expected, the presence of pBR327 had no effect on the levels of EPSP synthase in *aroA* cells. Cells carrying pMON12, a plasmid that does not complement the *aroA* deficiency, showed the same low level of EPSP synthase as the pBR327-containing control cells. In contrast, cells containing the *aroA*-complementing pMON2 and pMON4 plasmids showed 5- and 17-fold increases in EPSP synthase activity, respectively. The difference between the two is consistent with differences in the size of the *E. coli* DNA insert carried in each plasmid. Because the cloned DNA segments increased levels of EPSP synthase, we conclude that these segments contain the structural gene for the enzyme.

TABLE 2. EPSP synthase activity in extracts of plasmid-containing cells

Strain	Plasmid	EPSP synthase activity	
		Sp act (U/mg)	Relative activity
SR485	pBR327	0.5	<0.01
SR488	pBR327	76	1
SR485	pMON12	4	0.05
SR485	pMON2	400	5.25
SR485	pMON4	1,300	17.11

Cells carrying *aroA* plasmid are tolerant to glyphosate. Plasmids pBR322 and pMON4 were used to transform *E. coli* 594 to Amp resistance. The resultant colonies were the same size, and growth experiments in LB Amp and M9 Amp confirmed that both transformants had the same growth rate. The effects of glyphosate on 594/pBR322 and 594/pMON4 growth rates were then compared. Growth was carried out as described above. Glyphosate was present from t = 0. To obtain an estimate of the level of glyphosate tolerance conferred on 594 cells, the experiment was conducted to identify the maximum level of glyphosate at which 594/pBR322 cells would grow and the minimum level at which 594/pMON4 cells were inhibited. The results appear in Fig. 4.

The growth rate of 594/pBR322 (Fig. 4a) was inhibited approximately 50% by 0.1 mM glyphosate. Inhibition became more severe with increasing concentrations of glyphosate. Inhibition could be partially alleviated by the addition of 5 mM phenylalanine, 5 mM tyrosine, and 5 mM tryptophan. Readings at 71 h after initiation of growth showed that the 594/pBR322 cells can grow to saturation in 0.1, 0.5, 1, and 5 mM plus the aromatic acids, but growth stopped after one doubling in 5 mM glyphosate alone.

The results for growth of 594/pMON4 were different (Fig. 4b). During 8 h of treatment, the growth rate was minimally affected by 1 mM glyphosate and only reduced by 40% in 5 mM, or about the level seen in 0.1 mM in the control cells. The growth rate was more severely reduced in 10 and 20 mM. At 40 mM, growth was barely detectable. However, 71 h after treatment, the cells reached saturation at all concentrations except 20 and 40 mM, at which saturation was reached after 80 h. Thus, the glyphosate tolerance conferred by pMON4 can be overcome by increased concentrations of glyphosate, but it is not abolished even at 40

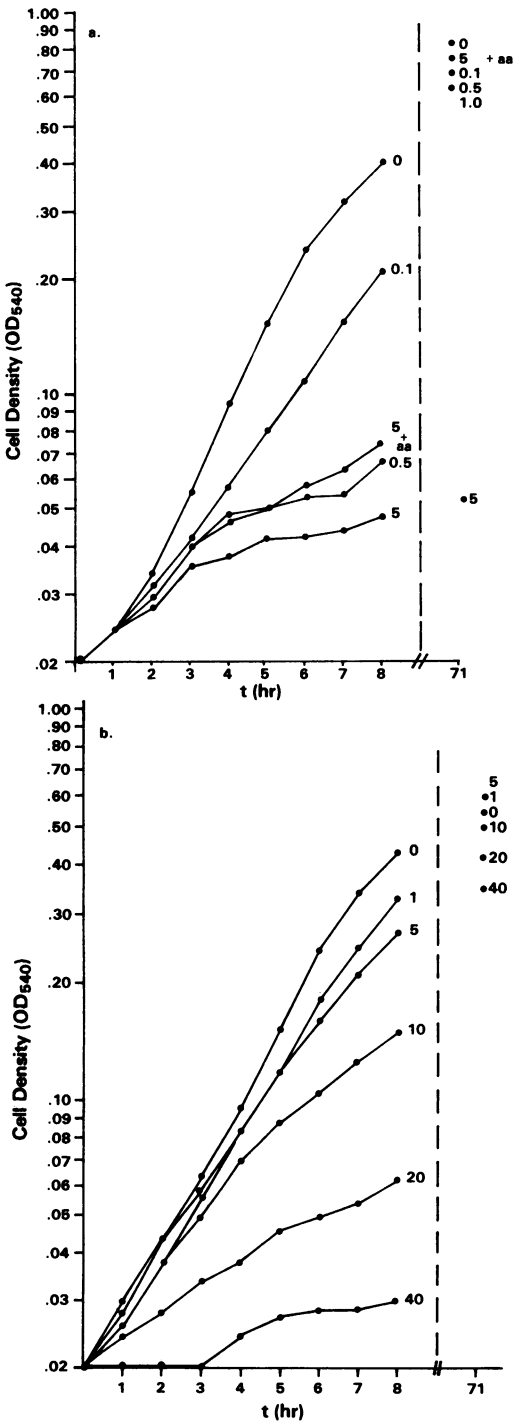

FIG. 4. Growth of plasmid-containing *E. coli* cells in the presence of glyphosate. The numbers next to the curves are glyphosate concentrations in millimoles per liter. The symbol aa indicates medium which contains the aromatic acids. (a) Strain 594 carrying pBR322. (b) Strain 594 carrying pMON4. OD_{540}, Optical density at 540 nm.

mM, which is a concentration eight times the amount required to severely inhibit growth of 594/pBR322 cells. Higher concentrations have not been tested to date.

Glyphosate tolerance is a selectable marker in *E. coli*. The glyphosate tolerance conferred by *aroA* plasmids can be demonstrated by colony formation on minimal plates containing glyphosate. For these experiments, a prototrophic host should be used for the *aroA* control plasmids as described above. Strain SR488 (*aroA*$^+$ *recA56*) was transformed with pMON2, pMON4, pMON12, and pBR327. Portions of the resulting Amp-resistant transformants were washed with M9 salts and spread on M9 Amp plates and M9 Amp plates containing 10 mM glyphosate. Colonies are detectable on M9 Amp plates after 2 days at 37°C. Although growth in the presence of glyphosate was retarded, colonies were readily visible on the 10 mM glyphosate plates after 3 days at 37°C. The relative efficiency of plating of control pBR327-containing cells was less than 10^{-6}. The same plating efficiency was seen for cells containing pMON12, a plasmid carrying a segment of DNA from the *aroA* region but not the *aroA* gene itself. In contrast, cells containing pMON2 or pMON4, which encode a complete *aroA* gene, formed colonies on 10 mM glyphosate plates with same efficiency seen on plates without the herbicide. Therefore, glyphosate tolerance is a selectable marker for transformation of *E. coli* cells.

DISCUSSION

These experiments demonstrate that glyphosate-tolerant *E. coli* is obtained simply by increasing the gene dosage of EPSP synthase. Direct selection for glyphosate tolerance was not required and was not imposed on the *aroA* plasmid containing 594/pMON4 cells. This is the first in vivo evidence that EPSP synthase is the target of glyphosate and the first example of a manipulable herbicide tolerance marker.

The level of relative resistance conferred by *aroA* amplification depends on the chosen endpoint for assessing inhibition. When growth rates over 8 h are compared, cells with increased EPSP synthase show the same growth rate in 5 mM glyphosate as control cells in 0.1 mM glyphosate. This indicates a 50-fold increase in tolerance. However, growth to saturation is a more important endpoint for indentification of a selectable marker. Because 5 mM glyphosate totally inhibits controls but *aroA* amplification allows growth to saturation in 40 mM glyphosate, the *aroA* plasmid increases the selectable tolerance to glyphosate by at least a factor of 8. This level of resistance closely parallels the level of EPSP synthase activity in these bacterial cells.

Although the segment of *E. coli* DNA carried in pMON4 is approximately 3.5 kb and could encode other genes, the *aroA* coding sequence probably is responsible for glyphosate resistance. This conclusion is based on the known effects of glyphosate on aromatic amino acid biosynthesis (9, 10) and on the reaction catalyzed by EPSP synthase demonstrated by Amrhein and his colleagues (1, 22) and in our laboratory. To determine whether amplification of EPSP synthase alone leads to glyphosate tolerance, deletion analysis of the pMON4 DNA insert is required. Deletion analysis of the *aroA* insert also will enable us to localize and manipulate the *aroA* coding region. Such work is currently in progress.

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